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DNA barcodes unite two problematic taxa: the meiobenthic *Boreohydra simplex* is a life-cycle stage of *Plotocnide borealis* (Hydrozoa: Aplanulata)

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Abstract

Genetic barcodes of arctic medusae and meiobenthic cnidarians have uncovered a fortuitous connection between the medusa *Plotocnide borealis* Wagner, 1885 and the minute, mud-dwelling polyp *Boreohydra simplex* Westblad, 1937. Little to no sequence differences exist among independently collected samples identified as *Boreohydra simplex* and *Plotocnide borealis*, showing that the two different forms represent a single species that is henceforth known by the older name *Plotocnide borealis* Wagner, 1885. The polyp form has been observed to produce bulges previously hypothesized to be gonophores, and the results here are consistent with that view. Interestingly, the polyp has also been reported to produce egg cells in the epiderm, a surprising phenomenon that we document here for only the second time. Thus, *P. borealis* produces eggs in two different life stages, polyp and medusa. This is the first documented case of a metagenetic medusozoan species being able to produce gametes in both the medusa and polyp stage. It remains unclear what environmental/ecological conditions modulate the production of eggs and/or medusa buds in the polyp stage. Similarly, sperm production, fertilization and development are unknown, warranting further studies.

Key words: polyp, medusa, metagenesis, bipolar distribution, meiofauna

Introduction

Medusozoan cnidarians often possess life cycles with adult swimming medusae that produce gametes and benthic polyp stages, sometimes as solitary individuals but usually as colonies. Because the life stages are found in distinct habitats, one often encounters either one or the other life stage in the field, and much historical taxonomic literature has dealt primarily with either medusae or polyps (hydroids within the class Hydrozoa), respectively. A lack of complete life cycle information has resulted in numerous cases where either the medusa or polyp stage is unknown for a given species, or higher taxon. In many cases, the two stages may be known, but have been described independently as separate species with the connection between them not yet recognized. Two approaches can address deficiencies in life cycle information for medusozoans: rearing animals and genetic barcoding of different life-cycle stages.

Rearing species in laboratory settings from either live polyps or medusae yields developmental information, and often behavioral observations, but it is not always achievable. Thus, genetic barcoding of large numbers of known polyps and medusae has the potential to efficiently unite life-cycle stages. Thus far, different life-cycle stages have been united through genetic barcoding for only a small number of cases, likely because the database of barcodes for both life forms is still relatively incomplete. Successful cases include Grossmann *et al.* (2013, 2014), who used genetic barcodes to link eudoxid stages to adult colony forms in the genus *Lensia*, and Miranda *et al.* (2010), who discovered that a putative hydrozoan polyp was actually a previously unknown life stage of a stalked

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medusa of the class Staurozoa. In the course of barcoding arctic medusae and meiobenthic cnidarians we have uncovered a fortuitous and unexpected connection between the medusa *Plotocnide borealis* Wagner, 1885 and the minute, mud-dwelling polyp *Boreohydra simplex* Westblad, 1937 (Fig. 1).

Materials and methods

Numerous samples of *Plotocnide borealis* and *Boreohydra simplex* were independently collected (Table 1). Samples of another solitary and potentially allied meiofaunal hydroid *Protohydra leuckarti* Greeff, 1870 were processed at the same time. These meiobenthic polyps were collected near the N.A. Pertsov White Sea Biological Station of the Moscow State University, Kandalaksha Bay, White Sea, Russia. *Boreohydra simplex* was collected from fine mud at depth 10–15 m; *Protohydra leuckarti* from muddy sand at the middle littoral zone. Surface sediment was filtered through a 150 µm sieve and examined in Bogorov chambers under a stereobinocular microscope. For histology, TEM and SEM examination the specimens were fixed in 2.5% glutaraldehyde in phosphate buffer (0.83 Osmol) at pH 7.3–7.4 (Millonig 1964).

Scanning electron microscopy. Specimens were dehydrated in a graded ethanol series (30%, 50%, 70%) and acetone, critical-point dried, sputter coated with gold or platinum, and examined with a Cam Scan scanning electron microscope.

Histology and transmission electron microscopy. Specimens were dehydrated, infiltrated and embedded in a mixture of Araldite 502/Embed-812 (Electron Microscopy Sciences, Catalog #13940) according to manufacturer's instructions. Blocks were sectioned using a Leica EM UC6 ultramicrotome. For histological study semithin sections ranging in thickness from 1.5 to 3 µm were stained with a mixture of toluidine blue and methylene blue and viewed with a Leica DM5000 B microscope. Photographs were obtained using Helicon Focus software. For ultrastructure examination, ultra-thin sections (50–70 nm) were stained with uranyl acetate and lead citrate and examined with a Jeol Jem-100B transmission electron microscope.

Genetic data and analyses. DNA extractions were carried out on single individuals using the AutoGenPrep 965 high-throughput DNA extraction robotic system (AutoGen) following the manufacturer's instructions for Whole Blood extraction. PCR amplification of mitochondrial 16S and nuclear 18S was carried out according to the protocols established in Collins *et al.* (2008), whereas COI was amplified and sequenced using the primers and protocol of Geller *et al.* (2013). Barcoding at multiple loci was done in light of the concerns raised by Lindsay *et al.* (2015) and because a lack of close relatives precluded identification of a "barcoding gap". Sequencing was accomplished using an Applied Biosystems 3730xl DNA Analyzer. Geneious 8 (Kearse *et al.* 2012) was used to assess quality of forward and reverse sequence reads, trim read ends, and assemble contigs. Each marker was aligned with all sequences of the hydrozoan subclass Hydroidolina (encompassing all Aplanulata, Capitata, Filifera, Leptothecata and Siphonophora) publically available from Genbank using the MAFFT plugin (Auto Algorithm; Katoh & Standley 2013) within Geneious, which was also used to measure genetic variation of the three markers reported in Table 2. Clustering analyses of the COI and 16S alignments (1623 and 1956 sequences, respectively) were conducted using the Geneious Treebuilder (neighbor joining with the HKY model of nucleotide evolution). Similarly, new 18S sequences were aligned with all publically available sequences of Hydroidolina (resulting in an alignment of 523 sequences). Using PhyML (Guindon *et al.* 2010), assuming the GTR model with estimated proportion of invariable sites and gamma distribution parameter, a maximum likelihood topology was generated and node support assessed by SH-like and bootstrap (420 replicates) indices (Fig. 1A). Topologies from each marker, along with the consensus topology of the ML bootstrap replicates of the 18S dataset are available at https://dx.doi.org/10.6084/m9.figshare.3406654.v3.

Results and discussion

Plotocnide borealis is a small medusa up to about 3 mm in bell height that is broadly distributed in shallow waters (to 200 m) of the Arctic Sea and recognizable by the presence of four short capitate tentacles, a simple tube-shaped manubrium encircled by gonads, and often oil-droplets deriving from the gastroderm that intrude into the thick apical mesoglea of the bell (Schuchert 2010). Recent surveys record the medusa in the plankton during the open

water season (August-September), but only in low numbers (Ershova *et al*. 2015). The species has a troubled taxonomic history, having been allied to several different families, and is presently *incertae sedis* within the hydrozoan suborder Capitata (Schuchert 2015). Similarly, the small (up to 1.5–3.0 mm long) spindle- or club-shaped solitary hydroid *Boreohydra simplex*, which lives in and on muddy sediments feeding upon nematode worms, has had a confused taxonomic history (Schuchert 2006). Possessing 3–4 short tentacles (sometimes 5 in the biggest specimens) below the mouth, nematocyst warts and a body that tapers into a mucus-covered muscular stalk, the species has been placed in its own family Boreohydridae or in the families Acaulidae, Corymorphidae and Candelabridae, all presently classified within the suborder Aplanulata (Schuchert 2015). *Boreohydra simplex* is the only species of the genus and has been reported to have a bipolar distribution.

Little to no sequence differences were observed among any samples identified as *Boreohydra simplex* and *Plotocnide borealis*, showing that the two different forms represent a single species that is henceforth known by the older name *Plotocnide borealis* Wagner, 1885 (see Schuchert (2006, 2010) for synonymy lists for both names). The cnidomes of polyp and medusa stages are similar, both possessing stenoteles and desmonemes, with the polyp stage also containing isorhizas (Bozhenova *et al.* 1989; Schuchert 2006, 2010).

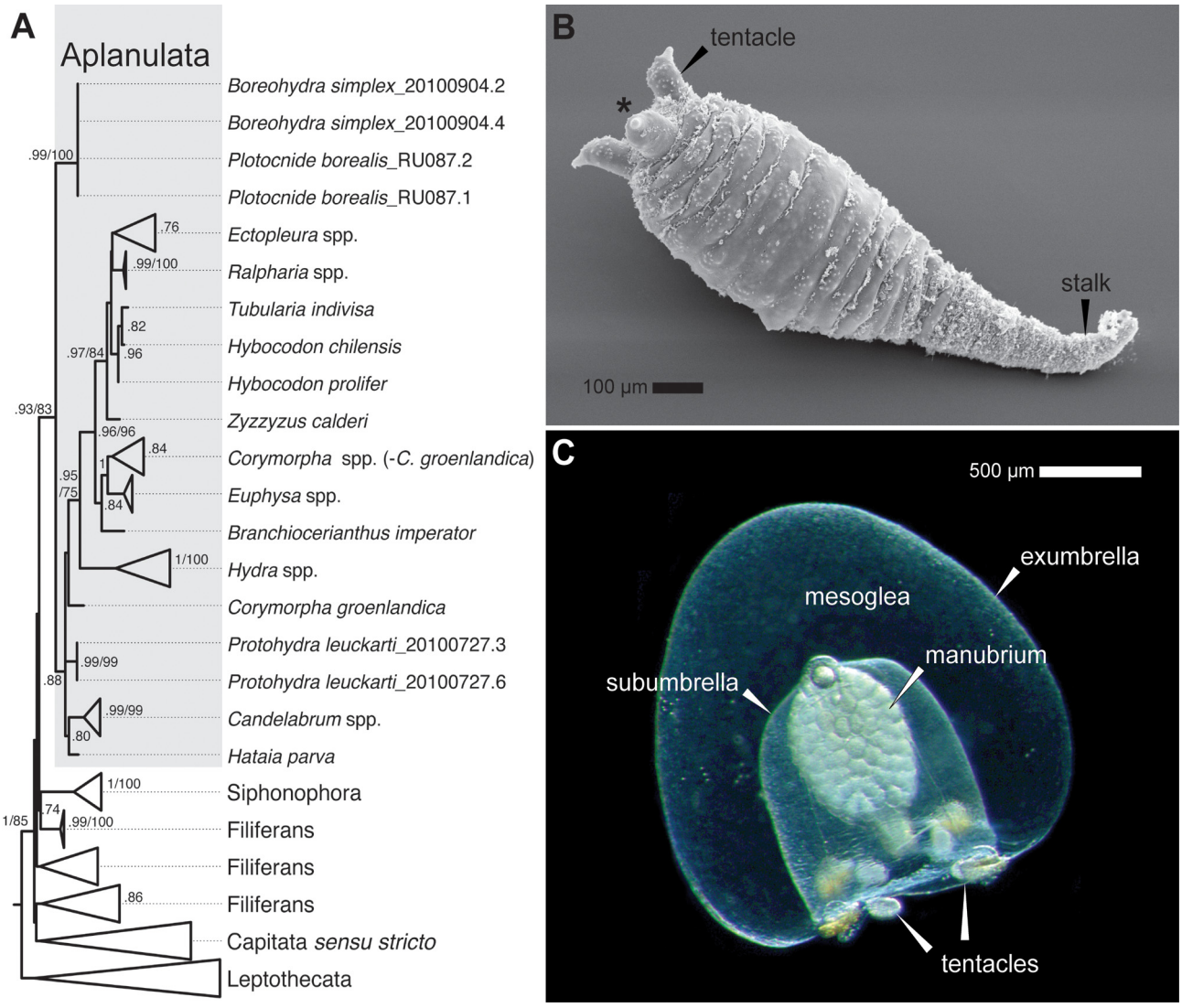

FIGURE 1. A. ML topology for all publically available 18S sequences (n=519) of Hydroidolina, showing the positions of *Plotocnide borealis* and *Protohydra leuckarti* within Aplanulata. SH-like branch support values are shown at the nodes, as well as bootstrap indices if exceeding 60. Uncollapsed topologies are provided as Supplementary Figures at https://dx.doi.org/10.6084/m9.figshare.3406654.v3. **B.** SEM of the polyp stage previously known as *Boreohydra simplex*, the mouth is marked by (*). **C.** Live image of the medusa stage of *Plotocnide borealis*, tentacles contracted.

TABLE 1. Samples used in this study, with GenBank Accession numbers for new sequences. Entire specimens were destroyed in the process of extracting DNA. Paravouchers for *Protohydra leuckarti* and *Boreohydra simplex* have been deposited in the Smithsonian National Museum of Natural History under USNM 1291104, 1291073, respectively.

Identification, field label	Locality	16S	COI	18S
Boreohydra simplex				
Boreohydra20100904.1	Russia, the White Sea, 66.53°N, 33.16°E, 10–15 m depth	KU721815	KU721804	
Boreohydra20100904.2	Russia, the White Sea, 66.53°N, 33.16°E, 10–15 m depth		KU721805	KU721830
Boreohydra20100904.3	Russia, the White Sea, 66.53°N, 33.16°E, 10–15 m depth	KU721816	KU721806	
Boreohydra20100904.4	Russia, the White Sea, 66.53°N, 33.16°E, 10–15 m depth	KU721817	KU721807	KU721831
Boreohydra20100904.5	Russia, the White Sea, 66.53°N, 33.16°E, 10–15 m depth		KU721808	
Boreohydra_SP	Russia, the White Sea, 66.53°N, 33.16°E, 10–15 m depth	KU721818		
Plotocnide borealis				
RU035	Chukchi Sea (Station CEN1) 70.795°N, 178.567°W, 29 m depth	KU721819	KU721809	
RU056	East Siberian Sea (Station WN11), 71.665°N, 179.5°E, 29 m depth	KU721820	KU721810	
RU087.1	Herold Valley, Chukchi Sea (Station HC15), 71.558°N, 175.790°W, 38 m depth	KU721821	KU721811	KU721832
RU087.2	Herold Valley, Chukchi Sea (Station HC15), 71.558°N, 175.790°W, 38 m depth	KU721822	KU721812	KU721833
Protohydra leuckarti				
Protohydra20100727.1	Russia, the White Sea, 66.55°N, 33.10°E, littoral zone	KU721823		
Protohydra20100727.2	Russia, the White Sea, 66.55°N, 33.10°E, littoral zone	KU721824		
Protohydra20100727.3	Russia, the White Sea, 66.55°N, 33.10°E, littoral zone	KU721825		KU721834
Protohydra20100727.4	Russia, the White Sea, 66.55°N, 33.10°E, littoral zone	KU721826		
Protohydra20100727.5	Russia, the White Sea, 66.55°N, 33.10°E, littoral zone	KU721827		
Protohydra20100727.6	Russia, the White Sea, 66.55°N, 33.10°E, littoral zone	KU721828	KU721813	KU721830
Protohydra20100727.7	Russia, the White Sea, 66.55°N, 33.10°E, littoral zone			
Protohydra20100727.8	Russia, the White Sea, 66.55°N, 33.10°E, littoral zone	KU721829		
Protohydra20100727.9	Russia, the White Sea, 66.55°N, 33.10°E, littoral zone			
Protohydra20100727.10	Russia, the White Sea, 66.55°N, 33.10°E, littoral zone		KU721814	

TABLE 2. Variation among and between genetic markers from *Boreohydra simplex* and *Plotocnide borealis* samples. *denotes that a single difference is fixed between samples of *Boreohydra simplex* and *Plotocnide borealis*.

Marker		Within *Boreohydra simplex*	Within *Plotocnide borealis*	Between *Boreohydra simplex* and *Plotocnide borealis*
COI				
	Number of Samples	5	4	9
	Sequence Length	633–654	582–654	582–654
	Percent Identity	100%	99.66–100%	99.66–100%
	Number of differences	0	0–2	0–2*
16S				
	Number of Samples	4	4	8
	Sequence Length	477–556	556	477–556
	Percent Identity	100%	100%	100%
	Number of differences	0	0	0
18S				
	Number of Samples	2	2	4
	Sequence Length	1465–1467	1471–1476	1465–1476
	Percent Identity	100%	100%	100%
	Number of differences	0	0	0

We now have a better, yet still incomplete, understanding of the reproduction and life cycle of this species. In the polyp form, *Plotocnide borealis* is capable of reproducing asexually via transverse fission (Westblad 1947) and/or longitudinally (according to our observations) during the entire year. From February to May, Westblad (1947) observed that *P. borealis* produces a small number (1–4) of globular bulges that resemble gonophores on the lower half of the polyp. However, he did not find any germ cells in these structures and hypothesized that the development of germ cells takes place at a later time. We can infer that gonophores bud off and develop into medusae that have been known as *P. borealis*, in which gonads develop around the stomach and upper portion of the manubrium.

A fascinating and complicating factor is that Nyholm (1951) observed what appeared to be solitary egg cells in the basal epiderm close to hydrocaulus in 2 of 150 polyp specimens sectioned. The putative egg cells lay entirely in the epidermal layer surrounded by an epiderm rich in cnidocysts, a condition similar to what is observed in species of *Hydra*. We also found several polyps from the same locality that possessed oocytes (up to 4 per specimen) in their epiderm (Fig. 2). The oocytes lay in the epiderm of the gastric region of the polyp in variable positions, sometimes closer to the stalk but also observed near the base of the tentacles. The cytoplasm of the oocyte is full of lipid droplets and vesicles filled with electron dense material (Fig 2E, 2F). We observed numerous invaginations of the outer membrane of the oocyte, which probably indicates that the oocyte uses surrounding ectodermal cells as nurse cells to increase its volume by incorporating cytoplasmic fragments from them, as is well known for *Hydra* (Honegger *et al*. 1989; Miller *et al*. 2000; Alexandrova *et al*. 2005). The invaginations are covered by a double membrane, thus incorporation is likely by phagocytosis rather than cell fusion.

It is remarkable that *P. borealis* produces eggs in two different life stages, polyp and medusa, which to our knowledge is unknown in any other species of Medusozoa. That said, variation in the site of gamete production is known within some species. For example, Aisenstadt & Polteva (1981) observed in a species of *Obelia* that germ cells originate from large amoeboid i-cells (up to 20 μm in length) in the epiderm of stem of the colony and then migrate first into gastroderm as the oocytes develop and then later along mesoglea toward sites of gonophore formation (Aisenstadt & Polteva 1981). The simultaneous potential for asexual and sexual reproduction of the polyp in *P. borealis* may be analogous to cases where the medusa stages of a species (e.g. *Proboscidactyla ornata* (McCrady, 1859), *Eucheilota paradoxica* Mayer, 1900, *Rathkea octopunctata* (M. Sars, 1835), etc.) can reproduce both sexually via gametes and asexually by budding off other medusae, polyps and/or frustules. In these cases, it has been shown that physical factors such as temperature and salinity impact whether gametes are produced or budding occurs (Werner 1958; Carré & Carré 1990). It seems reasonable to hypothesize that environmental

conditions might also influence the production of eggs or medusa buds in the polyp stage of *P. borealis* but detailed observations are lacking. Similarly, sperm production, fertilization and development are unknown, warranting further studies.

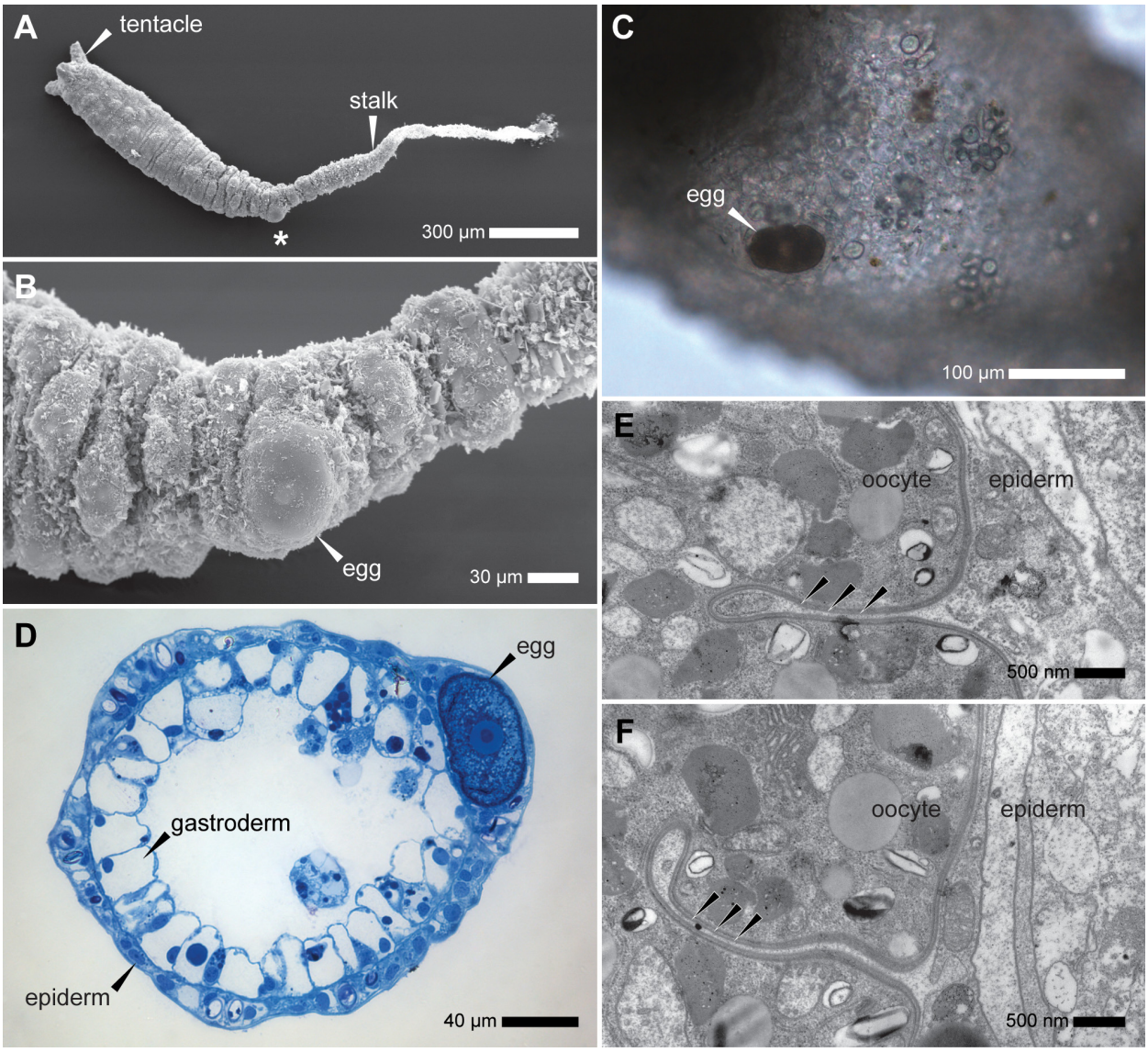

FIGURE 2. The egg of the polyp stage of *Plotocnide borealis*. **A.** SEM of the polyp with the egg, marked by (*). **B.** SEM of the egg, front view. **C.** Live image of the gastric region of the polyp with the egg. **D.** Transverse semi-thin section of the polyp stage stained with a mixture of toluidine blue and methylene blue clearly showing the egg. **E, F.** TEM of the oocyte wall, the invaginations of the outer membrane are marked by arrows.

Schuchert (2010) summarizes the distribution of the medusa *Plotocnide borealis* as Arctic circumpolar and northern boreal. First described as *Boreohydra simplex* from the fjords of the Scandinavian Atlantic coasts (Westblad 1937), the polyp stage of *P. borealis* has also been recorded from various north Atlantic localities, as well as South Georgia (Schuchert 2006), and more recently from the Sea of Japan (Sanamyan & Sanamyan 2012). Westblad (1953) hypothesized that *P. borealis* (as *B. simplex*) could even be cosmopolitan: distributed and known in polar and boreal areas where it reaches into the littoral zone at the most northern localities (the records from Iceland), whereas in lower latitudes it could be located much deeper in colder waters (Westblad 1953). The Southern Ocean observation of the polyp stage might be considered at odds with the distribution of the medusa as reported in Schuchert (2010). However, there is a single observation of *Plotocnide* sp. from New Zealand (Barnett 1985) and a record of *Plotocnide incertae* (Linko, 1900) from off the southern tip of Africa (Buecher *et al*. 2005). That said, Kramp (1942) suggested that *P. incertae* is most likely referable to a separate genus in the family Tubulariidae. Thus, to date there is just a single observation of a medusa identified to the genus *Plotocnide* and just

a single report of its polyp stage from the Southern Ocean, highlighting the need for further exploration of this region.

Finally, the phylogenetic position of *Plotocnide borealis* (as well as the meiofaunal polyp *Protohydra leuckarti*) within Hydrozoa is of some interest. As noted above, *Plotocnide borealis* is presently classified within Capitata, whereas *Boreohydra simplex* is classified within Aplanulata. Until recently, these two suborders were united within a single taxon known as Capitata, characterized by the presence of capitate tentacles. However, those capitates that lack a ciliated planula larval stage form a monophyletic assemblage now known as Aplanulata (see Nawrocki *et al*. (2013) for discussion). Our 18S analyses (Fig. 1A; supplementary figures at https://dx.doi.org/10.6084/m9.figshare.3406654.v3) suggest that both *Protohydra leuckarti* and *Plotocnide borealis* are members of Aplanulata. In particular, *Plotocnide borealis* is identified as the earliest known diverging lineage within Aplanulata, albeit with limited support. In agreement with our analysis, a recent study of complete mitochondrial genome sequences found *Plotocnide borealis* to be the earliest diverging lineage of Aplanulata (Kayal *et al*. 2015), although that study had relatively limited taxon sampling. Because life cycle variation (specifically loss of medusa stages) exists within Aplanulata, a lineage possessing both polyp and medusa stages at or near the base of the clade will inform character reconstructions as more taxon-rich and robust analyses emerge.

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